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Abstract: The fruit fly Drosophila melanogaster and the baker's yeast Saccharomyces cerevisiae are classic research model organisms that are also associated in nature, at least around vineyards. Sharing the same ephemeral fruit niche, winged Drosophila feed on immotile yeasts. That a yeast diet is essential for larvae development and saprophagous fruit flies are attracted to a suite of yeast volatiles has been well established over the last century. Recently, research has focussed on the potential mutual benefit of this interaction hypothesising yeasts also benefit via dispersal from ephemeral fruits. It now appears the concept of a co-evolved mutualism between yeasts and Drosophila has permeated the literature. However, until robust evidence regarding the evolution and maintenance of this yeast-fly association has been provided, we suggest there is no compelling evidence to reject the more simplistic null hypothesis that these interactions are due to exaptation, and not a mutualism driven by natural selection.

Commentary

DO YEASTS AND *DROSOPHILA* INTERACT JUST BY CHANCE?

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ABSTRACT

The fruit fly *Drosophila melanogaster* and the baker's yeast *Saccharomyces cerevisiae* are classic research model organisms that are also associated in nature, at least around vineyards. Sharing the same ephemeral fruit niche, winged *Drosophila* feed on immotile yeasts. That a yeast diet is essential for larvae development, and that saprophagous fruit flies are attracted to a suite of yeast volatiles, has been well established over the last century. Recently, research has focussed on the potential mutual benefit of this interaction hypothesising yeasts also benefit via dispersal from ephemeral fruits. It now appears that the concept of a co-evolved mutualism between yeasts and *Drosophila* has permeated the literature. However, until robust evidence regarding the evolution and maintenance of this yeast-fly association has been provided, we suggest there is no compelling evidence to reject the more simplistic null hypothesis that these interactions are due to exaptation, and not a mutualism driven by natural selection.

Keywords

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INTRODUCTION

The emergence of Ascomycota, Basidiomycota and Glomales, which comprise the three major groups of fungi, can be dated to 600-million years ago (Mya), and plant-fungus mutualisms are known to have occurred during early colonisation of land by terrestrial plants in the Ordovician about 460 Mya (Redecker *et al.* 2000), 60-million years before the first

land-bound insect evolved flight in the Devonian period. Within the Ascomycota, two independent lineages emerged from a metabolically aerobic ancestor about 500 Mya:

1) fission yeasts (Taphrinomycotina)

2) budding yeasts (Saccharomycotina).

Both lineages independently evolved a novel metabolic strategy (the Crabtree effect) where sugars are preferentially fermented even in the presence of oxygen (Dashko *et al.* 2014). The ability to propagate as facultative anaerobes, however, was modified during further diversification events within the Saccharomycotina subphylum over the last 200 Mya, leading to Crabtree-positive (preferential use of fermentation that can occur simultaneously with respiration) and Crabtree-negative (preferential respiration) species.

Yeasts are often associated with insects and plants (Chandler *et al.* 2012, Stefanini *et al.* 2012, Witzgall *et al.* 2012, Six 2013) and fermenting species quickly dominate in sugar-rich sources such as fruits, where they produce ethanol in the presence of oxygen (preferential fermentation) and other volatile compounds (Cordente *et al.* 2012). Human history is linked with the utilisation and refinement of food and beverage fermentation by microbes, with evidence of their preparation from rice, honey and fruit (hawthorn and/or grape) as early as 7000 BC (McGovern *et al.* 2004). Preferential fermentation is one of the most distinguishing features of *Saccharomyces* species, and this has actively been harnessed by humans to such a degree that certain lineages of this species are termed "domesticated" (Legras *et al.* 2007, Liti *et al.* 2009). *Drosophila* fruit flies of the subgenus *Sophophora* such as *D. melanogaster* are human commensals which may also co-inhabit the same ephemeral fruit niche as fermenting yeasts, and they cloud around wineries and often drown themselves in fermented foods, like wine and vinegar. *Drosophila* evolved in the mid-late Eocene (40 Mya) from a common

ancestor and further diversified in response to biogeographic events (Izumitani *et al.* 2016). *Drosophila* are generally saprophagous microbe-feeders although a trophic shift to herbivory evolved in the subgenus *Scaptomyza* about 20 Mya which might be linked to the loss or pseudogenisation of odour-receptors crucial for the recognition of yeast volatiles (Goldman-Huertas *et al.* 2015).

It is well documented that a yeast diet enhances fly fecundity and larvae development (Anagnostou *et al.* 2010, Rohlfs and Kurschner 2010, Matavelli *et al.* 2015) and while a few studies provide tentative evidence of a mutualism with specific fly and yeast isolates (Buser *et al.* 2014, Christiaens *et al.* 2014), the origin and extent of more general *Drosophila-Saccharomyces* interactions has not described: are they all mutualisms (see supplementary Table for definition of terms)? Have yeasts and flies generally coevolved as a result of selective pressures to optimise their life history traits and reproductive output? Or has any association arisen by chance from a coincidental combination of pre-existing adaptations (exaptation) of both partners in just a handful of specific situations?

Biochemistry of yeast volatile production and hypotheses for biological benefit

The evolution of the Crabtree effect allows most *Saccharomyces* yeasts to employ preferential alcoholic fermentation, even in the presence of oxygen, as powerful means of ecosystem engineering (Goddard 2008, see supplementary Table for definition of terms): in sugar-rich media, glucose is converted to cytotoxic ethanol, carbon dioxide and heat. Although respiration delivers more ATP to the organism, therefore increasing biomass production, preferential fermentation is ecologically successful. Preferential fermentation may act as an antagonistic strategy to both sabotage and outcompete other microorganisms as it allows ATP to be generated more rapidly, which translates to a greater growth rate, and

simultaneously creates a toxic, hot and alcoholic environment (Goddard 2008; Pfeiffer & Morley 2014). In addition, along with the predictions from niche-construction theory, Crabtree-positive yeasts are particularly well adapted not only to survive the hostile conditions they create but also to defend carbon resources from competitors as many species have evolved the ability to catabolise ethanol (Thomson *et al.* 2005; Pfeiffer & Morley 2014).

In addition to ethanol, yeasts also produce short to medium-chain alcohols (fusel alcohols) during assimilation of plant-based amino acids (Fig 1). Fusel alcohols are formed from fusel aldehydes via the Ehrlich pathway and can be further oxidised to organic acids (Hazelwood *et al.* 2008). These volatile organic compounds may be converted into more complex metabolites by the cell, with one example being alcohol acyl transferases (ATF, Fig 1) which use alcohols as precursors for the formation of esters at the expense of acetyl-CoA or fatty acyl-CoAs that are common intermediates of the primary sugar and fatty acid metabolism. Yeast volatiles may escape the cell and diffuse rapidly through air. Volatile ester biosynthesis and the formation of fusel-like aldehydes, alcohols and acids is not exclusive to yeasts and are common among fruits (El Hadi *et al.* 2013). However, yeasts alter the composition and concentrations of fruit volatiles to produce a different chemical signature (Cordente *et al.* 2012). Olfactory recognition of these esters and fusel volatiles is common in vertebrates as well as invertebrates feeding on fruit, which is not surprising considering that these compounds are a signal for food sources. Generally, several biological functions have been hypothesised for volatile ester production in yeasts, summarised by Saerens *et al.* (2010), and these suggest they act as metabolic ‘relief valves’, detoxification pathways, or fill an ecological function as infochemicals to promote dispersal by insects. To date, there is no clear evidence that allow these hypotheses to be robustly tested.

The effects of fungal secondary metabolites on insects can generally be classified into stimulants, attractants, deterrents and repellents (Holighaus & Rohlfs 2016) which constitute a variety of diverging biological functions. In addition, certain filamentous fungi such as *Aspergillus* respond to insect-grazing by induced production of mycotoxins, such as the polyketide sterigmatocystin, to successfully defeat their predators (Rohlfs 2015). Although this secondary metabolite is not volatile, polyketides are formed from acetyl-CoA precursors, the same substrate used by ATF for the formation of volatile esters. The enzymatic apparatus required for the biosynthesis of polyketides, however, has not naturally evolved in yeasts. To date, yeast secondary metabolites with insecticidal properties have not been identified whereas there is evidence for their role as insect attractants or repellents, especially in association with *Drosophila* (Hutner *et al.* 1937, Becher *et al.* 2012, Palanca *et al.* 2013, Scheidler et al. 2015).

The role of yeast volatiles in mediating *Drosophila* interactions

That *D. melanogaster* feasts on yeasts and that this microbial diet is crucial for larval development was first reported by Baumberger (1917) and later confirmed by Phaff (1956) who isolated yeasts from the alimentary canal of fruit flies. It is now established that yeast are an essential staple of *Drosophila* diet, and common life history traits of *D. melanogaster* such as survival, development time and adult body weight are influenced by both, yeast-species and yeast-biomass available to the larvae (Anagnostou *et al.* 2010). Further, yeast diversity was also shown to positively impact *Drosophila* life history traits not only by increasing larval development, possibly due to enhanced nutrition, but also by increasing larval survival rates in the presence of antagonistic filamentous fungi (Rohlfs & Kurschner 2010). Yeasts can detoxify mycotoxins (Hathout & Aly 2014), and this might add further

potential benefits for flies. Further, Stamps *et al.* (2012) observed that *D. melanogaster* larval activity increased yeast density while simultaneously reducing species diversity to increase yeasts that are more beneficial to larvae. This has been termed 'proto-farming' where growth rate survival and/or density of an edible species (yeast) increases at a locality due to the presence of a "farmer" (larvae).

Given the benefits derived from yeasts, it is unsurprising that *D. melanogaster* odour receptors respond to yeast volatiles (Hallem and Carlson 2004, 2006). Experiments conducted with a few strains of *S. cerevisiae*, show yeast volatiles can attract fruit flies, stimulate oviposition and affect mating success by increasing sexual receptivity in females (Becher *et al.* 2012, Gorter *et al.* 2016). Even in the absence of yeasts, fermentation-like volatiles can lure fruit flies (Stökl *et al.* 2010) and at least one case of specialised floral mimicry is described in a rainforest orchid which attracts a single drosophilid species (*Scaptodrosophila bangi*) for pollination (Martos *et al.* 2015). These examples of chemical mimicry of yeast volatiles by plants suggest a relationship between yeasts and flies that is sufficiently old and stable to have allowed secondary mimics to evolve.

Although several volatiles are likely to act as semiochemicals (Table 1, see supplementary Table for definition of terms), the evidence suggests the effectiveness of *Drosophila* attraction by *Saccharomyces* yeasts is not controlled by the presence or absence of single compounds but is a function of volatile ratios in combination with a fruit context (Arguello *et al.* 2013, Günther *et al.* 2015). Any yeast-fly interaction has to be considered as part of a tripartite relationship which includes fruit/plant, and this raises the question of to what extent the attractiveness of a particular yeast is contingent on the third plant partner. For example,

yeasts synthesise 3-methylbutanol from L-leucine via the Ehrlich-pathway and the corresponding acetate ester is known to mediate *D. simulans* attraction, but only when presented in a fruit context (Günther *et al.* 2015).

At first inspection the nature of the yeast-*Drosophila* association appears to be one of predation, i.e. it comprises benefit only for *Drosophila*. Usually organisms avoid predation, be it through defence (chemical or physical) or escape. Ethanol production by yeasts might have acted as a predator repellent ancestrally, but it cannot be regarded as an anti-predator adaptation against fruit flies today. Yeasts share the same ephemeral fruit niche with insects such as *Drosophila*. However, one key difference between these species is that yeasts are immotile and depend on dispersal via other agents to persist. Following this, one idea suggests that yeast volatile production is a means of chemical communication (see supplementary Table for definition of terms) with microbe-feeding flies, and this is beneficial as it increases the propensity of yeast dispersal to new habitats by flies (Saerens *et al*. 2010, Buser *et al*. 2014, Christiaens *et al*. 2014). If this were the case then the yeast-fly association would be bidirectional and resemble characters of mutualism, where both partners must receive increased fitness from the association, and not a simple predator-prey interaction where only one side of the association realises fitness increases (West *et al.* 2007). If this proposed mutualism was maintained by chemical communication then particular yeast volatiles will be under selection, and research suggests that volatile ester production, especially the acetate esters ethyl acetate and 3-methylbutyl acetate (Christiaens *et al.* 2014, Günther *et al.* 2015), is important for *Drosophila* attraction.

Are yeast-fly associations just chance?

The mutualism hypothesis articulated above appears intuitively attractive, but the evidence to support this is weak and sporadic. *D. melanogaster* and *S. cerevisiae* are significant model organisms in their own right and thus very good candidates to study interspecific interactions of microbes and insects. *D. melanogaster* is likely the best studied animal model which has been used for genetic research for over a century and *S. cerevisiae* was the first eukaryote to have its genome sequenced in 1996. Despite this, the ecological reality of these organisms is embedded in multitrophic networks, and any data generated from their analyses in isolation may not be representative:

First, correlations between attraction and yeast dispersal have only been shown for three *S. cerevisiae* genotypes with just two *Drosophila* isolates (one *D. simulans* and one *D. melanogaster*, Buser *et al.* 2014, Christiaens *et al.* 2014). Further, some *S. cerevisiae* genotypes are repulsive, and others have different levels of attractiveness, to at least two iso-female lines of *Drosophila* (one *D. simulans* and one *D. melanogaster*; Buser *et al.* 2014; Palanca *et al.* 2013), demonstrating that attraction is by no means a fixed trait. In addition, *S. cerevisiae*, is not abundant on fruit and rarely associated with *D. melanogaster* naturally (Goddard & Greig, 2015, Hoang *et al*. 2015, Lam & Howell 2015). Together this calls an evolved mutualism of these species into question. Other than *S. cerevisiae*, olfactory response of *D. melanogaster* was evaluated to a limited range of *Saccharomycetales* yeasts from other genera, such as *Hanseniaspora uvarum* and *Pichia kluyverii* (Palanca *et al* 2013, Hoang *et al*. 2015, Scheidler *et al* 2015), which are more commonly associated with *Drosophila* in nature (Dobzhanski *et al*. 1956, Hamby *et al*. 2012, Lam & Howell 2015). However, whether attraction corresponds with success in yeast dispersal for these is not known.

Second, one might be tempted to view a mutualism between yeasts and flies as underpinned by adaptation of the expression of *atf1* for acetate ester formation in yeasts and the corresponding odour receptor genes (*or43b, or47a, or85b*) in *Drosophila*. Chemical signals can evolve from cues (Steiger *et al.* 2011, see supplementary Table for definition of terms) but to date there are no compelling data to suggest that yeast volatile production has evolved to establish a mutualism. Further, in evolutionary terms, budding yeasts are hundreds of millions of years older than *Drosophila* fruit flies and we have little data concerning the history of the interaction between these two organisms. Despite their long-standing status as model organisms, genetic evidence of traits selected for as a result of their interactions in nature is scarce. Although several studies imply the coevolution of yeasts and flies (Stökl *et al.* 2010, Goldman-Huertas *et al.* 2015, Martos *et al.* 2015) mechanisms supporting their molecular evolution are yet to be elucidated. Odour receptors responding to yeast volatiles such as acetate esters are generally broadly tuned (Mansourian & Stensmyr 2015) conferring attraction to a range of chemically similar compounds. Therefore, volatiles that stimulate antennal responses and might influence behaviour are not strictly yeast-specific. Adaptive evolution has been described for the alcohol dehydrogenase (*Adh*) locus in *Drosophila* which is crucial for the detoxification and carbon-recycling of ethanol and at least three independent parallel-evolutionary events have resulted in novel protein functions of the ancestral gene between 2-30 Mya (Jones & Begun 2005). Ethanol tolerance is linked to ADH protein levels in *Drosophila* and differs drastically even between evolutionary sibling species such as *D. melanogaster* and *D. simulans* with the latter showing reduced ethanol tolerance and lower ADH-levels (Laurie *et al.* 1990). Ethanol adaptation has also been linked to acetic acid tolerance in *D. melanogaster* and was suggested as a mechanism to reduce environmental ethanol stress and allow colonisation of substrates altered through microbial infestation (Chakir *et al.* 1993). While differential adaptations to ethanol and acetic acid are likely to

impact yeast preference and *D. simulans* was repulsed by acetic acid (Günther *et al.* 2015), both sympatric *Drosophila* species are attracted to the Brewer's yeast *S. cerevisiae*.

Third, the ecological relationship of *Saccharomyces* and *Drosophila* is far from exclusive. *Saccharomyces* are not only associated with fruit flies, but other insects including bees and wasps (Goddard *et al*. 2010; Stefanini et al. 2012). While specific flies prefer particular yeasts over others (Palanca *et al.* 2013, Buser *et al.* 2014, Scheidler *et al.* 2015) and differ in their attraction to infested substrates (Matavelli *et al.* 2015, Date *et al.* 2017), only a few genera of yeasts are consistently associated with fruit fly populations. Frequently isolated yeasts include *Candida, Pichia, Hanseniaspora, Metschnikowia, Torulaspora* but rarely *Saccharomyces* (Hamby *et al.* 2012, Stamps *et al.* 2012, Buser *et al.* 2014, Lam and Howell 2015). None of these yeasts are exclusively associated with *Drosophila,* and distributions of these yeasts have been shown to be more strongly influenced by *Drosophila* diet rather than fly species in at least fifteen common *Drosophila* populations (Chandler *et al.* 2012). Although flies might discriminate for some yeasts, alternative species are likely to compensate when the favourite food is absent (Dobzhansky *et al.* 1956). Further, it has long been known that flies are not only associated with yeasts but also bacteria (Baumberger 1917). The bacterial gut commensals *Lactobacillus* and *Acetobacter* are indirectly involved in yeast-fly associations by enhancing the flies' appetite for dietary yeasts in response to amino acid deprivation (Leitao-Goncalves *et al.* 2017). Although bacterial volatiles generally trigger a different set of odour receptors in the fly, *Drosophila* is also attracted to volatile amines released during bacterial decomposition of organic matter (Min *et al*. 2013). Throughout the literature a subset of volatiles (Table 1) has been suggested to act as semiochemicals, mediating fruit fly attraction. These volatiles are not only produced by a range of yeasts but also by bacteria and some fruits:

Small quantities of acetic acid are released during fermentation by yeasts, but the accumulation of acetic acid is more commonly a result of oxidative fermentation of ethanol by acetic acid bacteria. Although ambiguous in its role as fruit fly attractant, acetic acid can be a very effective lure for the vinegar fly *D. melanogaster*, enhance mating success and stimulate oviposition (Joseph *et al.* 2009, Cha *et al.* 2012, Gorter *et al.* 2016). Other key volatiles for *Drosophila* attraction, namely 3-methyl butanol and acetoin also comprise major volatiles produced by the human skin microbiota, for example by *Staphylococcus epidermidis* (Verhulst *et al.* 2009). Therefore, *Drosophila* attraction to a particular substrate is likely the result of microbial activity, involving yeast and/or bacteria and both microbes are potentially dispersed by the fly. Lastly, O'Conner *et al.* (2014) hypothesised that the adaptive radiation of *Drosophila* populations endemic to Hawaii was linked to plant colonisation with symbiotic yeasts, and that flies might be radiating with the microbes rather than host plants themselves. Recent studies testing host plant specificity of cactophilic *D. majovensis* state that olfactory preference for the plant species can shift in response to plant-microbe and microbe-microbe interactions thus emphasising the importance of each partner to volatile composition in this three-way interaction (Date *et al.* 2017). While yeasts might play a role in *Drosophila* phylogenetic diversification, there are no data to suggest that yeast speciation coevolved with *Drosophila* or flying insects in general, as yeasts evolved before flying insects emerged.

Although a few studies have provided evidence that interactions between specific *Drosophila* and *S. cerevisiae* isolates may be classed as a facultative mutualism, there is no evidence to support the claim that this may be generalised further. At present there is no compelling evidence to reject the null hypothesis that general interactions between *Drosophila* and *Saccharomyces* are either coincidental, fortuitously resulting from pre-existing traits, i.e. this interaction is an exaptation. At most the evidence supports a one-way interaction in terms of

benefits for *Drosophila*. If yeast volatile production were an adaptation acting to attract insects, then this would have to be classed as a generalist approach, targeting a broad range of flying vectors (Table 1) including wasps, moths and beetles in addition to *Drosophila*. Taking a generalist approach in enhancing odours to attract a variety of vectors rather than relying on one species for dispersal seems a strategy that selection would more readily operate on, but is a more elusive idea to test, and there is no evidence that flying insects other than *Drosophila* derive fitness benefits from their association with yeasts.

It is desirable to consider experiments that could rigorously test hypotheses regarding the nature of interactions between yeast and flies. The facultative nature of these associations makes such experiments hard to conduct. Ultimately these would need to assay the fitness benefits and costs of specific associations. Buser *et al*. (2014) have done this in a limited way and found different extents of yeast fitness (measured by dispersal) among isolates. First one would need to assay multiple combinations of fly and yeast genotypes to evaluate the space over which both fly and yeast fitness were increased: if this were large, a more general claim for a mutualistic interaction would be supported. This is a huge task. Another approach is to evaluate the degree to which these interaction and fitness benefit traits are malleable. If these traits change readily over a few generations, this suggests that such associations are not acted on strongly by selection: i.e. they are ephemeral, just like the fruit that yeasts and *Drosophila* temporarily co-inhabit.

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CAPTIONS

Figure 1: Volatile production in yeast. Linking Ehrlich-pathway with fatty acid biosynthesis and preferential fermentation (in the presence of oxygen). ATF: Alcohol acyl transferase; CA: Acetyl-CoA carboxylase

Figure

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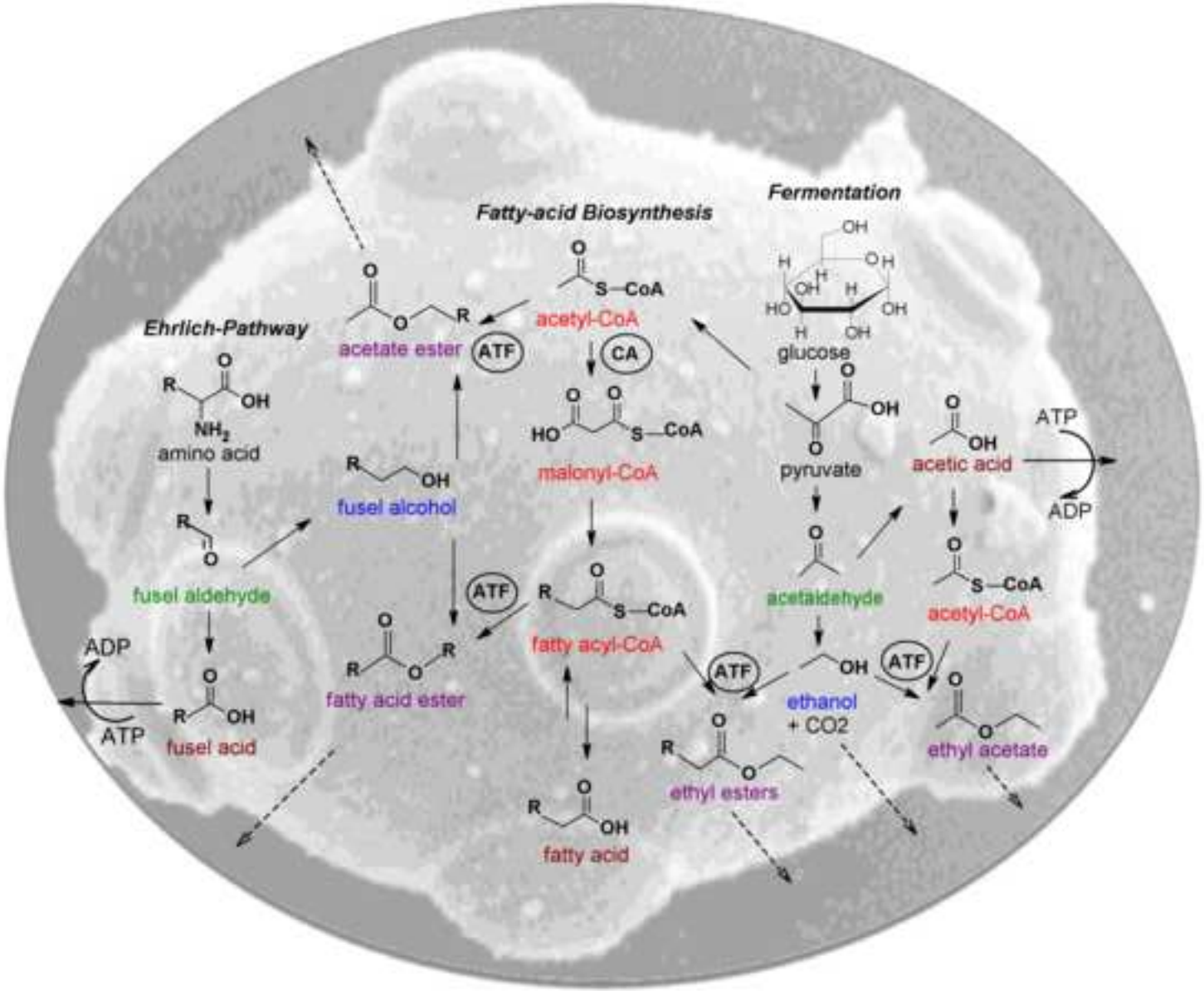

Table

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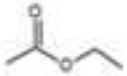
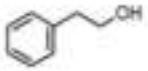
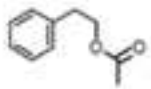
Table 1: Yeast volatiles and their potential role as semiochemicals for insect attraction.

Yeast volatiles	acetic acid (ethanoic acid)	ethanol (ethyl alcohol)	ethyl acetate (ethyl ethanoate)	3-methylbutanol (isoamyl alcohol)	3-methylbutyl acetate (isoamyl acetate)	3-hydroxybutanone (acetoin)	2-phenylethanol (2-phenethyl alcohol)	2-phenylethyl acetate (2-phenethyl acetate)
								
Suggested as semiochemical for attracting *Drosophila*	✓ [2; 4; 9; 11]	✓ [2; 4; 17]	✓ [4; 5]	✓ [1; 2]	✓ [4; 5; 10]	✓ [1; 2; 4]	✓ [1; 2; 4; 15]	✓ [15]
Insects responding	[12] Synergistically enhances attraction to semiochemicals for moths (*Lepidoptera: Noctuidae*)	[13] *Carpophilus humeralis* (sap beetle)	[13] *Carpophilus humeralis* (sap beetle) and [14] *C. hemipterus* (dried fruit beetle); [8] *Graphania mutans* (cutworm)	[13] *Carpophilus humeralis* (sap beetle) and [14] *C. hemipterus* (dried fruit beetle); [16] *Cydia pomonella* (codling moth); [8] *Graphania mutans* (cutworm); [7] *Vespula sp.* (wasp); [6] *Syrphidae* (hoverflies); [3] *Dendroctonus frontalis* (pine beetle)	[16] *Cydia pomonella* (codling moth); [3] *Dendroctonus frontalis* (pine beetle)	[13] *Carpophilus humeralis* (sap beetle) and [14] *C. hemipterus* (dried fruit beetle)	[16] *Cydia pomonella* (codling moth); [8] *Graphania mutans* (cutworm); [7] *Vespula sp.* (wasp); [6] *Syrphidae* (hoverflies); [3] *Dendroctonus frontalis* (pine beetle)	[16] *Cydia pomonella* (codling moth); [3] *Dendroctonus frontalis* (pine beetle)

References [1] Arguello et al. 2013; [2] Becher et al. 2012; [3] Brand et al. 1977; [4] Cha et al. 2012; [5] Christiaens et al. [6] Davis and Landolt 2013; [7] Davis et al. 2012; [8] El-Sayed et al. 2005; [9] Gorter et al. 2016; [10] Günther et al. 2015; [11] Joseph et al. 2009; [12] Landolt et al. 2013; [13] Nout and Bartelt 1998; [14] Phelan and Lin 1991; [15] Stökl et al. 2010; [16] Witzgall et al. 2012; [17] Zhu and Fry 2015

e-component

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